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Rice kernel phenolic content and its relationship with antiradical efficiency†‡

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Abstract: Plant phenolics exert beneficial effects on human health and may also prevent oxidative deterioration of food. Two field experiments were carried out for characterising phenolics in rice. The first assay was conducted in 1999 and 2000 in Beaumont, TX and included five light-brown, two purple and 10 red pericarp coloured cultivars. 'Bran colour' was highly statistically significant for both bran phenolic concentration and antiradical efficiency ($p < 0.001$). 'Year' and its interaction with bran colour were not significant for the analysed traits, suggesting that seasonal differences and their interactions may not affect phenolic content or antiradical efficiency. The accessions ranged from 3.1 to 45.4 mg gallic acid equivalents (GAE) g^{-1} bran and from 10.0 to 345.3 μM trolox equivalents (TE) g^{-1} bran for total phenolic content and antiradical efficiency respectively. The light-brown bran genotypes exhibited the lowest values for phenolic content and antiradical efficiency, whereas red bran ones displayed *ca* 10 times higher total phenolic content and more than 50 times higher tannin content than light-brown ones. The two purple lines showed either low or high values for the studied traits. Antiradical efficiency of rice bran extracts was highly positively correlated with total phenolic content ($r = 0.99^{***}$), suggesting that phenolics are the main compounds responsible for the free radical-scavenging activity in rice bran extracts. In the second field experiment (Stuttgart, AR, 2001 and Beaumont, TX, 2000), 133 coloured rice cultivars were analysed for total phenolic content in whole grain. The accessions showed a large variation for total phenolics, ranging from 0.69 to 2.74 mg GAE g^{-1} grain. The data confirmed previous results suggesting bran colour as the main factor affecting phenolic concentration in rice kernel and seasonal effects and their interactions as not significant. The results also confirm that within red and purple bran groups can be found the highest phenolic concentrations in rice kernel.
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Keywords: rice; phenolics; antioxidant; tannins; antiradical efficiency; DPPH

INTRODUCTION

Rice (*Oryza sativa* L) grain contains several classes of antioxidants, including phenolic compounds, tocols and γ-oryzanol. Antioxidants reportedly are protective against oxidative damage, which has been implicated in a range of diseases, including cancer and cardiovascular disease.[1] They are also one of the principal ingredients that protect food quality by preventing oxidative deterioration of lipids.[2]

Recent studies on rice have demonstrated that its potential health benefits appear to be related to its pericarp colour. Ling *et al*[3] found that liver reactive oxygen species, aortic malondialdehyde and the area of atherosclerotic plaque were significantly lower in rabbits fed rice with red or purple bran (ie pericarp) than in those fed rice with white bran. Moreover, Toyokuni *et al*[4] reported a protective effect against renal lipid peroxidation in rats fed rice with dark coloured bran over rice with light bran colour. The higher antioxidative effect of coloured rice as compared with that from white or light bran coloured rice may be explained by differences in the phenolic content or other components in the rice kernel. In fact, phenolic concentration in rice appears to be strongly related to bran colour, with cultivars with red and purple bran showing up to 20 times higher concentrations as compared with those with white or light-brown bran.[5] Furthermore, Oki *et al*[6] determined the DPPH radical-scavenging activity in three rice cultivars differing in pericarp colour (white, black and red). They found that coloured rice has notably higher radical-scavenging activity than white rice, the polymeric procyanidins being the major components responsible for that activity. In spite of their potential beneficial health effects, little is known about phenolics in rice, the staple diet for much of the world's population. The present study was performed to assess the potential of rice as a source of

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phenolic antioxidants by evaluating a rice germplasm collection, to characterise rice phenolic composition into low- and high-molecular-weight compounds and to examine the relationship between rice phenolics and radical-scavenging activity.

EXPERIMENTAL

Chemicals

Folin–Ciocalteau's phenol reagent, ethanolamine, gallic acid, Sephadex LH20, 4-morpholinepropanesulphonic acid sodium salt (MOPS), sodium azide, dimethyl sulphoxide (DMSO), (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (trolox) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were purchased from Sigma-Aldrich Corp (St Louis, MO, USA). HPLC-grade methanol (MeOH) and acetone were obtained from Fisher Scientific (Houston, TX, USA). Non-denatured ethanol (EtOH, 200 proof, HPLC-grade) was purchased from Pharmco Products Inc (Brookfield, CT, USA). Reagent-grade, bacteria-free water was produced by a Barnstead Nanopure II deionisation system (Dubuque, IA, USA). Glucose determination reagent kit (glucose oxidase, peroxidase and 4-aminoantipyrine) was obtained from Megazyme International Ireland Ltd (Bray, Co Wicklow, Ireland).

Field experiment I (Beaumont, TX, 1999 and 2000)

A field study was conducted in 1999 and 2000 in Beaumont, TX and included 17 rice accessions differing in pericarp colour (light-brown, purple and red). Classification into bran colour groups was based on descriptions made by the USDA, ARS, National Genetic Resources Program, Germplasm Resources Information Network (GRIN), National Germplasm Resources Laboratory, Beltsville, MD (http://www.ars-grin.gov/cgi-bin/npgs/html/desclist.pl?75). The genotypes were analysed for total extractable phenolic and low- and high-molecular-weight phenolic contents in the bran and for their antiradical efficiency. The plants were cultivated in single plots, arranged in a completely randomised design, using cultural practices common to the region. The plots consisted of six rows, 3.5 m long, spaced 15 cm apart. The within-row spacing was about 10 cm. The plots were kept continuously flooded at about 10 cm of standing water. At maturity the plants were threshed by hand, the grains were dehulled and all broken, diseased and immature kernels were removed.

Field experiment II (Stuttgart, AR, 2001 and Beaumont, TX, 2002)

A second field experiment was performed to estimate the variation for total phenolic content in a large rice germplasm collection consisting of 133 coloured rice cultivars. The coloured rice collection was grown in Stuttgart, AR in 2001 and in Beaumont, TX in 2002 and included US unadapted plant material selected for bran colour diversity. The genotypes were analysed for total extractable phenolic content in the whole kernel. In addition, the total extractable phenolic content in the bran was determined in those genotypes from which enough grain material was obtained for the milling process (>50 g). The accessions were cultivated using the same design and practices used in the first field experiment.

Rice milling procedure

About 50 g of dehulled kernels were milled using a McGill mill #1 for 30 s with an 858 g weight in position 12 or 6 for long- and medium-grain types respectively. The bran fraction was collected and sieved through an 840 µm sieve. Bran samples were stored in a freezer (−20 °C) under nitrogen until analysis. Surface lipid content was determined by refluxing 5 g of milled rice with petroleum ether in a Goldfish extraction apparatus (Lab comco, MO, USA) for 30 min. The solvent was collected and evaporated; per cent surface lipid content was calculated as the mass of the extracted lipid divided by the beginning total milled rice mass. This measurement was used to ensure that all samples were milled within a similar range in degree of milling (ie <0.5% surface lipid content).

Determination of total extractable phenolics in whole rice kernels

Brown rice was ground with a Cyclone sample mill (0.4 mm screen, UDY Corp, Boulder, CO, USA). About 200 mg of ground rice was extracted overnight (17 h) with 2.5 ml of MeOH. Samples were mixed briefly with a Vortex mixer, once at the beginning and once at the end of the extraction time. They were then centrifuged for 5 min at $3822 \times g$. The supernatant was filtered using a 1 µm glass syringe filter and then analysed for total extractable phenolics by the Folin–Ciocalteu assay.[7] The extracts were diluted in deionised water at 1:23 (v/v) to make a final volume of 1.2 ml. Folin–Ciocalteu reagent (500 µl) and 0.5 M ethanolamine (1 ml) were added to the diluted extracts. The samples were mixed and the absorbance at 600 nm was measured after exactly 30 min. Phenolic contents were calculated using a calibration curve developed with gallic acid standards (2–25 $\mu g\, ml^{-1}$). Results were expressed in mg gallic acid equivalents (GAE) g^{-1} whole grain (wet weight basis). Single analysis was performed for this measurement.

Determination of total extractable phenolics in rice bran

About 100 mg of rice bran was extracted overnight (17 h) with 8 ml of MeOH on a Hoefer red rotor mixer platform (Hoefer Pharmacia Biotech Inc, San Francisco, CA, USA) on a setting of 5. Samples were then centrifuged for 5 min at $3822 \times g$ and the methanolic extract was filtered through a 1 µm syringe glass filter. Filtered extracts were diluted in deionised water according to their bran colour as follows (v/v)—white, light and speckled brown bran at 1:11, purple and red bran cultivars at 1:59—to make a final volume of 1.2 ml. Phenolic determination was performed as described above. Results were expressed in mg GAE g^{-1} bran (dry weight basis) after determining the moisture content. For that, about 400 mg of rice bran was dried for 2 h at 105 °C and the moisture content was determined by weight difference. Moreover, since rice endosperm contains a very low concentration of phenolics as compared with bran (Goffman FD and Bergman CJ, unpublished data), differences in the amount of endosperm contaminating bran after milling will influence the bran phenolic concentration. The values were therefore corrected to 15% w/w starch content in the bran using the following formula:

$$\text{corrected phenolic content (mg GAE g}^{-1}) = \text{phenolic content 'as is' (mg GAE g}^{-1}) \times (100\% - 15\%)\,(100\% - \%\text{ starch of sample})^{-1}$$

Analysis was done in duplicate.

Separation and analysis of low-molecular-weight phenolics and tannins

A 200 µl aliquot of each of the filtered methanolic extracts from the previous bran phenolic determination was dried in vacuum and redissolved in 750 µl of EtOH by placing the sample in an ultrasonic bath for 5 min. The extracts were then applied to small glass columns—adapted Pasteur pipettes—containing 40 mg of Sephadex LH20 each. Absolute ethanol was added to each column (3 × 1 ml). The eluted fraction—low-molecular-weight phenolics—was collected, dried in vacuum and redissolved in 500 µl of MeOH. The columns were then rinsed with 70% acetone (3 × 1 ml) to elute the high-molecular-weight phenolics or tannins. The collected fraction was dried under vacuum and redissolved in 500 µl of MeOH. The low-molecular-weight phenolic fraction was diluted in deionised water at 1:11 (v/v). The tannin fraction was diluted as follows (v/v)—light-brown bran at 1:11, red and purple bran cultivars at 1:23—to make a final volume of 1.2 ml. Phenolic determination was performed as described above. Analysis was done in duplicate.

Antiradical efficiency assay

The antiradical efficiency of rice methanolic extracts was determined by monitoring the reduction in the absorbance (515 nm) of a 25 mg l^{-1} methanolic solution of DPPH after adding an aliquot of the extract. DPPH solution was prepared daily and kept protected from light in amber glass. Rice extracts were added to the 25 mg l^{-1} DPPH solution at (v/v) 1:149 (light-brown bran cultivars) or 1:599 (purple and red bran cultivars), mixed well with a glass rod and immediately scanned at 515 nm. Absorbance values were registered during 30 min and plotted against time, the resulting curves being then integrated. The integration values of DPPH after adding the extracts were compared with those obtained from a blank solution of DPPH (zero antiradical activity). Integration values were expressed in µM trolox equivalents (TE) g^{-1} bran (dry weight basis) after developing a calibration curve using different trolox solutions (0.2–4 mM in MeOH), which were added to the 25 mg l^{-1} DPPH solution at 1:149 (v/v). The values were corrected to 15% w/w starch content in the bran as follows:

$$\text{corrected antiradical efficiency (µM TE g}^{-1}\text{ bran}) = \text{antiradical efficiency 'as is' (µM TE g}^{-1}) \times (100\% - 15\%)\,(100\% - \%\text{ starch of sample})^{-1}$$

Analysis was carried out in duplicate.

Starch content analysis

A method by McClearly *et al*[8] for starch dermination was modified for rice bran analysis. Rice bran samples (50 mg) were weighed into test tubes and wet with 200 µl of aqueous ethanol (80% v/v). DMSO (2 ml) was immediately added and the tubes were stirred with a vortex mixer and placed in boiling water for 5 min. After that, 3 ml of thermostable α-amylase (100 U ml^{-1}) in MOPS buffer (50 mM, pH 7.0) containing calcium chloride (5 mM) and sodium azide (0.02% w/v) was immediately added. The tubes were vigorously stirred with a mixer and further incubated for 6 min, being stirred three times during incubation. The samples were then transferred to a bath at 50 °C, and 4 ml of sodium acetate buffer (200 mM, pH. 4.5) was added, followed by 100 µl of amyloglucosidase (200 U ml^{-1}). They were then stirred and incubated for 30 min. After incubation the samples were adjusted to 10 ml volume and mixed thoroughly. An aliquot of 200 µl of this solution was centrifuged at 2867 × g for 10 min and diluted to 600 µl with distilled water. A 3 ml aliquot of glucose determination reagent (12 000 U l^{-1} glucose oxidase, 650 U l^{-1} peroxidase and 0.4 mM 4-aminoantipyrine) was added and the samples were held at 50 °C for 20 min. The absorbance at 510 nm was registered for each sample, and starch contents were calculated using a calibration curve developed with glucose standards. Starch content was expressed as % w/w (dry weight basis). Analysis was performed in duplicate.

Statistical analysis

In the first experiment, cultivars were classified according to their bran colour, and data were subjected to a factorial analysis of variance (factorial ANOVA), 'year' and 'bran colour' being considered as factors. 'Bran colour' was further partitioned into variation due to comparisons among colour groups. In addition, cultivar means were compared by calculating the least significant difference (LSD) for each variable at $p = 0.05$ after performing a one-way ANOVA. A factorial ANOVA was also performed for the second experiment and included the same factors. Means for colour groups were compared using Duncan's multiple range test. All statistical analyses and correlations were performed with SAS software (Statistical Analysis System Institute, Cary, NC, USA).

RESULTS AND DISCUSSION

Field experiment I (Beaumont, TX, 1999 and 2000)

Table 1 shows the analysis of variance for total extractable phenolic and low- and high-molecular-weight phenolic contents in the bran of 17 rice cultivars and for the antiradical efficiency of their methanolic extracts. 'Bran colour' was highly statistically significant for all analysed traits ($p < 0.001$). The data confirm our previous results[5] which suggested that phenolic content and antiradical efficiency are both strongly related to pericarp colour. The variation due to bran colour was further partitioned into comparisons among colour groups. More than 85% of the variation for bran colour was due to differences between light-brown cultivars and coloured bran ones (purple and red). Differences between purple and red cultivars were only significant for tannin content at $p = 0.05$. 'Year' and its interaction with bran colour were not significant for the analysed traits, except for the interaction 'year × purple vs red', suggesting that seasonal differences and their interaction with bran colour may not affect both phenolic content and antiradical efficiency. 'Year' and 'year × bran colour' effects were very small for tannin content, indicating that tannin concentration in the bran is independent of seasonal changes and their interaction with bran colour.

The means over two years for phenolic concentration and antiradical efficiency in the 17 studied cultivars are presented in Table 2. The accessions showed extremely diverging values for the analysed traits: total phenolic content and antiradical efficiency ranged from 3.1 to 45.4 mg GAE g^{-1} bran and from 10.0 to 345.3 μM TE g^{-1} bran respectively. The light-brown bran genotypes exhibited the lowest values for total phenolic and low- and high-molecular-weight phenolic contents as well as antiradical efficiency (means 3.4, 2.9 and 0.5 mg GAE g^{-1} bran and 11.5 μM TE g^{-1} bran respectively). Their phenolic composition was characterised by a high proportion of low-molecular-weight phenolics (mean 86.5%). On average, red bran genotypes displayed approximately 10 times higher total phenolic content (34.5 vs 3.4 mg GAE g^{-1}) and more than 50 times higher tannin content (25.8 vs 0.5 mg GAE g^{-1}) than light-brown ones. Contrarily to light-brown bran cultivars, red bran accessions were distinguished by a high proportion of tannins (mean 65%). The two purple cultivars showed large differences for phenolic concentration and composition as well as for their antiradical efficiency, suggesting that a large variation may be found for these traits within this colour group.

It should be considered that, by using methanol as the extraction solvent, antioxidants other than phenolics are also extracted, including tocopherols, tocotrienols and γ-oryzanol. These antioxidants also exhibit an antiradical effect against DPPH. We have found that antiradical efficiency was highly positively correlated with total phenolic content ($r = 0.99^{***}$), which suggests that phenolics are the main compounds responsible for the free radical-scavenging activity in rice bran methanolic extracts. This observation is supported by the fact that phenolic compounds exhibit up to four times higher antiradical activity against DPPH as compared with α-tocopherol.[9] Further investigations are needed to evaluate the antiradical efficiency of individual rice phenolic compounds.

Field experiment II (Stuttgart, AR, 2001 and Beaumont, TX, 2002)

Table 3 presents the mean squares of the ANOVA for total phenolic content in whole grain of 133

Table 1. Mean squares of the analysis of variance for total extractable phenolic, low-molecular-weight phenolic and tannin contents and antiradical activity in the bran of 17 rice accessions (Beaumont, TX, 1999 and 2000)

Source	df	Low-P[a] (mg GAE g^{-1})	Tannin[b] (mg GAE g^{-1})	Total-P[c] (mg GAE g^{-1})	ARE[d] (μM TE g^{-1})
Year	1	44.27^{NS}	1.48^{NS}	61.33^{NS}	17320.51^{NS}
Bran colour	2	246.64***	2168.38***	3557.77***	193122.42***
Light-brown vs purple and red (I)	1	445.42***	3789.61***	6842.67***	377764.39***
Purple vs red (II)	1	47.86^{NS}	547.14*	272.88^{NS}	8480.44^{NS}
Year*bran colour	2	44.66^{NS}	0.90^{NS}	50.98^{NS}	5424.17^{NS}
Year*(I)	1	2.85^{NS}	1.69^{NS}	8.61^{NS}	5192.34^{NS}
Year*(II)	1	86.46*	0.10^{NS}	93.36^{NS}	5656.00^{NS}
Error	28	10.66	49.22	87.35	5097.89

Significance: * $p = 0.05$; *** $p = 0.001$; ^{NS} not significant.
[a] Low-molecular-weight phenolic content.
[b] Tannin content.
[c] Total extractable phenolic content.
[d] Antiradical efficiency.

Table 2. Means over two years for total extractable phenolic, low-molecular-weight phenolic and tannin contents and antiradical activity in the bran of 17 rice cultivars (Beaumont, TX, 1999 and 2000)

Cultivar	Bran colour	Low-P[a]	Tannin[b]	Total-P[c]	ARE[d]
		(mg GAE g^{-1})			(μM TE g^{-1})
Dawn	Light-brown	2.7	0.4	3.1	11.3
Dixiebelle	Light-brown	3.0	0.4	3.5	10.0
IARI 6626	Light-brown	3.2	0.5	3.8	12.8
Panbira	Light-brown	2.7	0.5	3.2	10.2
WIR 605	Light-brown	3.0	0.4	3.5	13.1
242	Purple	20.6	24.8	45.4	345.3
HB-1	Purple	7.5	1.1	8.6	56.3
Achhame	Red	9.4	22.4	31.8	227.7
Banjul	Red	9.3	29.0	38.2	276.0
Chokoto	Red	9.2	30.3	39.5	274.7
Gbeingbein	Red	8.0	29.3	37.3	261.7
IARI 6627	Red	14.9	29.0	43.9	312.3
Kakani 2	Red	10.5	22.0	32.5	210.5
Kun Shan Wu Shan	Red	13.6	25.2	38.9	267.0
Ngasein	Red	9.4	16.6	26.0	197.8
Pokkali	Red	9.1	30.8	40.2	285.4
Srav Ankor	Red	8.9	23.0	31.9	199.0
LSD ($\alpha = 0.05$)		5.8	10.6	13.5	119.8

[a] Low-molecular-weight phenolic content.
[b] Tannin content.
[c] Total extractable phenolic content.
[d] Antiradical efficiency.

Table 3. Mean squares of the analysis of variance for total extractable phenolic content in the kernels of 133 rice accessions (Stuttgart, AR, 2001 and Beaumont, TX, 2002)

Source	df	Total-P[a] (mg GAE g^{-1})
Year	1	6.17*
Bran colour	2	62.71***
Year*bran colour	2	1.91^{NS}
Error	260	0.95

Significance: * $p = 0.05$; *** $p = 0.001$; ^{NS}not significant.
[a] Total extractable phenolic content.

rice cultivars. 'Bran colour' was highly statistically significant for total phenolics ($p < 0.001$), whereas 'year' and 'year × bran colour' were not significant. Thus the data confirm the results obtained from the previous experiment, which suggested that seasonal effects and their interaction with bran colour do not influence phenolic contents.

The means of the bran colour groups differed significantly at $p = 0.05$ for total phenolics (Table 4), the lowest values being found in the brown bran group and the highest in the purple one (0.69 and 2.74 mg GAE g^{-1} grain respectively). Considering the fact that purple rices also displayed a large coefficient of variation (CV = 65.52%), it appears to be possible to find cultivars exhibiting even higher phenolic contents within this colour group. In fact, Ryu *et al*[10] studied just 10 black rice varieties and found anthocyanin contents ranging from 0 to 4.93 mg g^{-1}. Brown bran rices displayed a large variation (CV = 92.10%), with total phenolic contents falling into the low–medium range, whereas purple and red groups showed genotypes with low to high total phenolic levels. However, the classification into brown and red bran groups is based on a visual estimation and can therefore lead to misclassifications. Only four of the 22 brown bran cultivars had values above the rest of the genotypes (from 19.4 to 33.4 mg GAE g^{-1} bran, the rest showing values below 5 mg GAE g^{-1} bran), which suggests that those brown bran cultivars may be misclassified as brown and actually belong to the red bran group. The results also indicate that rices having purple or red pericarp do not necessarily contain a high phenolic concentration in the kernel.

The analysis of total phenolics in the bran (Beaumont, TX, 2002 only) is presented in Table 5. All colour groups significantly differed for total phenolic contents ($p < 0.05$), except for the comparison

Table 4. Means and ranges over two years for total extractable phenolic content in the kernels of 133 rice accessions, classified according to their bran colour (Stuttgart, AR, 2001 and Beaumont, TX, 2002)

Bran colour	n	Mean total-P[a] (mg GAE g^{-1})	CV (%)	Maximum	Minimum
				(mg GAE g^{-1})	
Brown	34	0.69c	92.10	2.46	0.25
Purple	13	2.74a	65.52	5.35	0.69
Red	86	2.13b	35.95	4.24	0.34

[a] Total extractable phenolic content. Different letters within the column indicate significant differences at $p = 0.05$ (Duncan's multiple range test).

Table 5. Means and ranges for total extractable phenolic content in the bran of 97 rice accessions, classified according to their bran colour (Beaumont, TX, 2002)

Bran colour	n	Mean total-P[a] (mg GAE g^{-1})	CV (%)	Maximum (mg GAE g^{-1})	Minimum (mg GAE g^{-1})
Brown	22	7.36c	122.31	33.38	1.90
Light-brown	12	3.39c	19.66	4.88	2.37
Purple	13	22.88b	74.51	50.32	4.76
Red	50	32.11a	21.95	47.19	19.95

[a] Total extractable phenolic content. Different letters within the column indicate significant differences at $p = 0.05$ (Duncan's multiple range test).

between light-brown and brown groups. When these results are compared with those presented in Table 2, it can be observed that the mean bran phenolic contents of colour groups fall consistently into similar ranges in both experiments. Light-brown bran cultivars exhibited a small range of variation (CV = 19.7%) and very low total phenolic contents (mean 3.4 mg GAE g^{-1} bran). Brown bran cultivars showed a low mean total phenolic content (7.36 mg GAE g^{-1} bran), with some cultivars displaying a medium total phenolic content. As indicated before, this may be caused by pericarp colour misclassification. Red and purple bran cultivars exhibited the highest total phenolic contents. These results confirm that within red and purple bran groups can be found the highest phenolic concentrations in rice bran.

In addition, a strong correlation was found between the total phenolic content of kernels and that in bran ($r = 0.93^{***}$). This suggests that it is possible to select for higher or lower phenolic content in the bran by analysing phenolic contents in whole grain (regardless of kernel dimensions), for which sample preparation is less time-consuming.

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